

We claim:

1. A modified pneumolysin polypeptide having attenuated hemolytic activity wherein said modified pneumolysin polypeptide is obtained by:

- a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid molecules in host cells;
- b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
- c) identifying the modified pneumolysin polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.

2. A modified properly-refolded pneumolysin polypeptide having attenuated hemolytic activity comprising an amino acid sequence of type 14 pneumolysin wherein at least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.

3. The modified pneumolysin polypeptide of claim 2, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.

4. A modified pneumolysin polypeptide according to claim 3, comprising at least one amino acid substitution in the amino acid sequence of Formula I ^{SEQ ID NO. 3} at residue positions 61, 148, or 195 or the combination of substitutions at residue positions 33, 46, 83, 239 and 257, said Formula I comprising

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(Formula I)

	Met	Ala	Asn	Lys	Ala	Val	Asn	Asp	Phe	Ile	Leu	Ala
	1				5					10		
5	Met	Asn	Tyr	Asp	Lys	Lys	Lys	Leu	Leu	Thr	His	Gln
		15						20				
	Gly	Glu	Ser	Ile	Glu	Asn	Arg	Phe	Ile	Lys	Glu	Gly
	25				30						35	
	Asn	Gln	Leu	Pro	Asp	Glu	Phe	Val	Val	Ile	Glu	Arg
10				40				45				
	Lys	Lys	Arg	Ser	Leu	Ser	Thr	Asn	Thr	Ser	Asp	Ile
		50					55				60	
	Ser	Val	Thr	Ala	Thr	Asn	Asp	Ser	Arg	Leu	Tyr	Pro
				65						70		
15	Gly	Ala	Leu	Leu	Val	Val	Asp	Glu	Thr	Leu	Leu	Glu
		75						80				
	Asn	Asn	Pro	Thr	Leu	Leu	Ala	Val	Asp	Arg	Ala	Pro
	85				90					95		
	Met	Thr	Tyr	Ser	Ile	Asp	Leu	Pro	Gly	Leu	Ala	Ser
20				100					105			
	Ser	Asp	Ser	Phe	Leu	Gln	Val	Glu	Asp	Pro	Ser	Asn
		110					115				120	
	Ser	Ser	Val	Arg	Gly	Ala	Val	Asn	Asp	Leu	Leu	Ala
				125						130		
25	Lys	Trp	His	Gln	Asp	Tyr	Gly	Gln	Val	Asn	Asn	Val
			135					140				
	Pro	Ala	Arg	Met	Gln	Tyr	Glu	Lys	Ile	Thr	Ala	His
	145				150						155	
	Ser	Met	Glu	Gln	Leu	Lys	Val	Lys	Phe	Gly	Ser	Asp
30				160					165			
	Phe	Glu	Lys	Thr	Gly	Asn	Ser	Leu	Asp	Ile	Asp	Phe
		170				175					180	
	Asn	Ser	Val	His	Ser	Gly	Glu	Lys	Gln	Ile	Gln	Ile
				185						190		
35	Val	Asn	Phe	Lys	Gln	Ile	Tyr	Tyr	Thr	Val	Ser	Val
			195					200				
	Asp	Ala	Val	Lys	Asn	Pro	Gly	Asp	Val	Phe	Gln	Asp
	205				210						215	
	Thr	Val	Thr	Val	Glu	Asp	Leu	Lys	Gln	Arg	Gly	Ile
40				220					225			
	Ser	Ala	Glu	Arg	Pro	Leu	Val	Tyr	Ile	Ser	Ser	Val
		230					235					240
	Ala	Tyr	Gly	Arg	Gln	Val	Tyr	Leu	Lys	Leu	Glu	Thr
				245						250		
45	Thr	Ser	Lys	Ser	Asp	Glu	Val	Glu	Ala	Ala	Phe	Glu
			255					260				
	Ala	Leu	Ile	Lys	Gly	Val	Lys	Val	Ala	Pro	Gln	Thr
	265				270						275	

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	Glu	Trp	Lys	Gln	Ile	Leu	Asp	Asn	Thr	Glu	Val	Lys
				280					285			
	Ala	Val	Ile	Leu	Gly	Gly	Asp	Pro	Ser	Ser	Gly	Ala
	290						295					300
5	Arg	Val	Val	Thr	Gly	Lys	Val	Asp	Met	Val	Glu	Asp
				305						310		
	Leu	Ile	Gln	Glu	Gly	Ser	Arg	Phe	Thr	Ala	Asp	His
			315					320				
10	Pro	Gly	Leu	Pro	Ile	Ser	Tyr	Thr	Thr	Ser	Phe	Leu
	325					330					335	
	Arg	Asp	Asn	Val	Val	Ala	Thr	Phe	Gln	Asn	Ser	Thr
			340					345				
	Asp	Tyr	Val	Glu	Thr	Lys	Val	Thr	Ala	Tyr	Arg	Asn
	350					355					360	
15	Gly	Asp	Leu	Leu	Leu	Asp	His	Ser	Gly	Ala	Tyr	Val
				365					370			
	Ala	Gln	Tyr	Tyr	Ile	Thr	Trp	Asn	Glu	Leu	Ser	Tyr
		375						380				
20	Asp	His	Gln	Gly	Lys	Glu	Val	Leu	Thr	Pro	Lys	Ala
	385					390					395	
	Trp	Asp	Arg	Asn	Gly	Gln	Asp	Leu	Thr	Ala	His	Phe
			400					405				
	Thr	Thr	Ser	Ile	Pro	Leu	Lys	Gly	Asn	Val	Arg	Asn
	410						415				420	
25	Leu	Ser	Val	Lys	Ile	Arg	Glu	Cys	Thr	Gly	Leu	Ala
				425					430			
	Trp	Glu	Trp	Trp	Arg	Thr	Val	Tyr	Glu	Lys	Thr	Asp
		435					440					
	Leu	Pro	Leu	Val	Arg	Lys	Arg	Thr	Ile	Ser	Ile	Trp
30	445					450					455	
	Gly	Thr	Thr	Leu	Tyr	Pro	Gln	Val	Glu	Asp	Lys	Val
				460					465			
	Glu	Asn	Asp									
	470											

35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

A B

~~SEQ ID NO: 3~~

no period.

- 35 5. The modified pneumolysin according to claim 4, wherein a single amino acid substitution is made and the substituted amino acid is selected from the group consisting of proline or hydroxyproline for position
- 40 B 61; lysine, arginine or histidine for position 148 and leucine, glycine, alanine, isoleucine or valine for position 195.
6. The modified pneumolysin according to claim 3, wherein the substituted amino acids are selected from

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B

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the group consisting of serine, threonine, asparagine, glutamine, tyrosine or ~~cysteine~~ ^{cysteine} for positions 33, 46 and 83; lysine, arginine or histidine for position 239 and leucine, glycine, alanine, isoleucine or valine for position 255.

7. Modified pneumolysin polypeptide pNVJ1.
8. Modified pneumolysin polypeptide pNVJ20.
9. Modified pneumolysin polypeptide pNVJ22.
10. Modified pneumolysin polypeptide pNVJ45.
- 10 11. Modified pneumolysin polypeptide pNVJ56.
12. Modified pneumolysin polypeptide pNV103.
- B 13. Modified pneumolysin polypeptide pNV207.
14. Modified pneumolysin polypeptide pNV111.
15. Modified pneumolysin polypeptide pNV211.
- 15 16. A recombinant nucleic acid molecule encoding a modified type 14 pneumolysin polypeptide wherein at least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.
- 20 17. The recombinant nucleic acid molecule according to claim 16 comprising the following pneumolysin nucleic acid sequence:

25	ATGGCAAATA AAGCAGTAAA TGA	TTTATA CTAGCTATGA	40
	ATTACGATAA AAAGAACTC TTGACCCATC	AGGGAGAAAG	80

	TATTGAAAAT	CGTTTCATCA	AAGAGGGTAA	TCAGCTACCC	120
	GATGAGTTTG	TTGTTATCGA	AAGAAAGAAG	CGGAGCTTGT	160
	CGACAAATAC	AAGTGATATT	TCTGTAACAG	CTACCAACGA	200
	CAGTCGCCTC	TATCCTGGAG	CACTTCTCGT	AGTGGATGAG	240
5	ACCTTGTTAG	AGAATAATCC	CACTCTTCTT	GCGGTCGATC	280
	GTGCTCCGAT	GACTTATAGT	ATTGATTTCG	CTGGTTTGGC	320
	AAGTAGCGAT	AGCTTCTCTC	AAGTGGAAGA	TCCCAGCAAT	360
	TCAAGTGTTT	GCGGAGCGGT	AAACGATTTC	TTGGCTAAGT	400
	GGCATCAAGA	TTATGGTCAG	GTCAATAATG	TCCCAGCTAG	440
10	AATGCAGTAT	GAAAAAATCA	CGGCTCACAG	CATGGAACAA	480
	CTCAAGGTCA	AGTTTGTTTC	TGACTTTGAA	AAGACAGGGA	520
	ATTCTCTTGA	TATTGATTTT	AACTCTGTCC	ATTCAGGCGA	560
	AAAGCAGATT	CAGATTGTTA	ATTTTAAGCA	GATTTATTAT	600
	ACAGTCAGCG	TAGACGCTGT	TAAAAATCCA	GGAGATGTGT	640
15	TTCAAGATAC	TGTAACGGTA	GAGGATTTAA	AACAGAGAGG	680
	AATTTCTGCA	GAGCGTCCTT	TGGTCTATAT	TTCGAGTGTT	720
	GCTTATGGGC	GCCAAGTCTA	TCTCAAGTTG	GAAACCACGA	760
	GTAAGAGTGA	TGAAGTAGAG	GCTGCTTTTG	AAGCTTTGAT	800
	AAAAGGAGTC	AAGGTAGCTC	CTCAGACAGA	GTGGAAGCAG	840
20	ATTTTGGACA	ATACAGAACT	GAAGGCGGTT	ATTTTAGGGG	880
	GCGACCCAAG	TTCGGGTGOC	CGAGTTGTAA	CAGGCAAGGT	920
	GGATATGGTA	GAGGACTTGA	TTCAAGAAGG	CAGTCGCTTT	960
	ACAGCAGATC	ATCCAGGCTT	GCCGATTTC	TATACAACTT	1000
	CTTTTTTACG	TGACAATGTA	GTTGCGACCT	TTCAAAATAG	1040
25	TACAGACTAT	GTTGAGACTA	AGGTTACAGC	TTACAGAAAC	1080
	GGAGATTTAC	TGCTGGATCA	TAGTGGTGCC	TATGTTGCC	1120
	AATATTATAT	TACTTGGAAT	GAATTATCCT	ATGATCATCA	1160
	AGGTAAGGAA	GTCTTGACTC	CTAAGGCTTG	GGACAGAAAT	1200
	GGGCAGGATT	TAACGGCTCA	CTTTACCACT	AGTATTCCTT	1240
30	TAAAAGGGAA	TGTTTCGTAAT	CTCTCTGTCA	AAATTAGAGA	1280
	GTGTACCGGG	CTTGCTTGGG	AATGGTGGCG	TACGGTTTAT	1320
	GAAAAAACCG	ATTTGCCACT	AGTGCGTAAG	CGGACGATTT	1360
	CTATTTGGGG	AACAACCTCT	TATCCGCAGG	TAGAAGATAA	1400
	GGTAGAAAAT	GAC	(SEQ ID NO: 1)		1413

35 and wherein said nucleic acid sequence comprises one or more of the nucleotide substitutions selected from the group consisting of:

A-50→G, G-54→T, T-181→C, A-196→T and T-302→C;

40 A-122→G, A-514→G, T-583→A and A-764→G;

A-187→T, T-380→A, A-382→C and T-443→A;

T-98→C, T-137→C, T-248→C, T-717→A and A-770→G;

a

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T-134→C, A-305→G, A-566→G and T-583→G;

T-583→G;

T-583→A;

T-443→A;

and

T-181→C.

18. The recombinant nucleic acid molecule of claim 16 as contained in a vector such as a plasmid, cosmid, bacteriophage or yeast artificial chromosome.

19. A microorganism comprising the nucleic acid molecule of claim 16.

20. The microorganism according to claim 19, wherein the microorganism is selected from the group consisting of bacteria, yeast, mammalian or insect cells.

21. The microorganism according to claim 20, wherein the microorganism is *E. coli*.

B 22. The modified pneumolysin polypeptide of ^{claim 2}~~claim 1~~, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

[Signature] 23. ~~The modified pneumolysin conjugate of claim 22, wherein the polysaccharide is from a bacteria selected from the group consisting of a Haemophilus influenzae type b; meningococcal group A, B or C; group B streptococcus types Ia, Ib, II, III, V or~~

VIII and pneumococcal.

24. A vaccine comprising at least one pneumolysin polypeptide of claim 1 and a pharmaceutically acceptable carrier.
- 5 25. The vaccine according to claim 24, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.
- 10 26. The vaccine according to claim 25, wherein the polysaccharide is derived from a bacteria selected from the group consisting of Haemophilus influenzae type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus serotypes Ia, Ib, II, III, V, or VIII; or one or more of serotypes 15 1-23 of *S. pneumoniae*.
- 20 27. A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to claim 1 in the presence of complement.
28. The method according to claim 27, wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate wherein the polysaccharide is a bacterial capsular polysaccharide.
- 25 29. A method for immunization of mammals comprising administering the vaccine of claim 24 to said mammals.
30. A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and

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add